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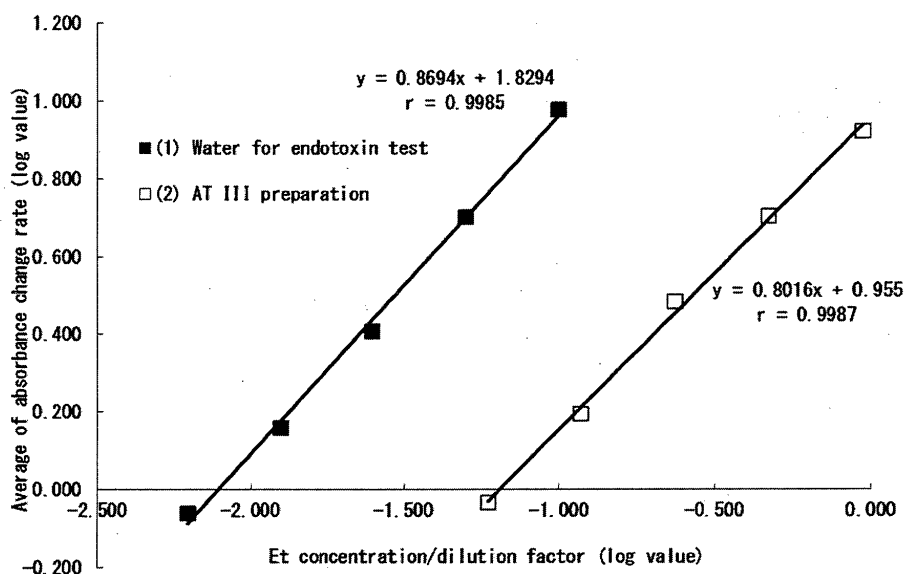
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(54) **PRETREATING AGENT FOR ANTITHROMBIN III FOR USE IN LIMULUS TEST, AND PRETREATMENT METHOD**

(57) To provide means for reducing reaction interference observed when AT III is subjected to limulus test, whereby the limulus test of antithrombin III can be carried out at high accuracy. Reaction interference observed

when AT III is subjected to limulus test can be reduced by subjecting AT III to a protein inactivation treatment in the co-presence of a divalent metal salt.

[Fig. 3]



Description

Technical Field

[0001] The present invention relates to a pre-treatment agent for antithrombin III which is to be subjected to a detection test for a target substance (e.g., endotoxin) employing a limulus reagent; to a limulus reagent kit including the pre-treatment agent; to a method for preliminarily treating antithrombin III and a method for measuring a target substance (e.g., endotoxin) by use of a limulus reagent, using these pre-treatment agent and limulus reagent kit.

[0002] In the present invention, the following abbreviations may be employed.

Et: endotoxin

BG: (1→3) - β -D-glucan

AT III: antithrombin III

Background Art

[0003] Endotoxin is a lipopolysaccharide which is present in the cell wall outer membrane of Gram-negative bacteria and is known as a strong pyrogen. Endotoxin is also known to cause various pathological conditions including not only fever but also shock and the like. Therefore, detection and quantitation of endotoxin is essential in pharmaceutical products such as an injection preparation, water, medical devices, and the like, from the viewpoint of ensuring safety of pharmaceutical products and the like.

[0004] As has been known, a horseshoe crab undergoes blood clotting when it is infected with a Gram-negative bacterium. This phenomenon has been conventionally employed for the detection of endotoxin. That is, endotoxin can be determined by use of blood corpuscle extracts of horseshoe crab (i.e., limulus amebocyte lysate, hereinafter referred to as "LAL"). This method is called "limulus test," which employs a cascade reaction of a variety of proteins (all serine proteases) present in LAL, which reaction occurs via contact between endotoxin with LAL.

[0005] AT III is an anti-coagulant in blood and is used for the treatment of disseminated intravascular coagulation (DIC) or the like. Since AT III is a serine protease inhibitor, it inhibits the activities of proteins which are present in LAL and which are involved in the cascade reaction (Non-Patent Document 1). Thus, in some cases, a certain type of a specimen inhibits a limulus reagent. Such an inhibitory action may be avoided by diluting a specimen (Non-Patent Document 2). However, AT III exhibits a very strong inhibitory action on a limulus reagent, and therefore, in order to carry out limulus test of an AT III preparation (injection), the preparation must be diluted at a dilution factor of 64 or higher (Non-Patent Document 2). Therefore, in consideration of the detection sensitivity of a limulus reagent (i.e. the lowest endotoxin concentration on the calibration curve), subjecting an AT III preparation to limulus test has been considered to be difficult (Non-Patent Document 2). Actually, even at present, detection of endotoxin in specimens each containing AT III is performed through the rabbit pyrogen test (Non-Patent Document 1).

[0006] Non-Patent Document 1 discloses a method for determining Et in an AT III preparation, in which method the AT III preparation is 100-fold diluted; the diluted product is heated at 70°C for 20 minutes as a pre-treatment; the pretreated product is reacted with LAL; and Et is detected through the light scattering method. A technical feature of the method resides in employment of the light scattering method by means of a light scattering measurement apparatus instead of turbidimetric assay by means of a spectrophotometer, in order to realize a high sensitivity in Et detection. However, similar to the aforementioned case, the method requires dilution of a specimen at high dilution factor, and involves at least the following problems.

(1) Spectrophotometers are widely employed as a measurement apparatus for limulus test, and a light scattering measurement apparatus must be additionally provided and used for carrying out the light scattering method.

(2) The Japanese Pharmacopoeia specifies only turbidimetric assay and chromogenic assay as photometric assay methods of Et, and is silent to a light scattering method.

[0007] Patent Document 1 discloses an Et and BG assay method for biological samples, the method comprising bringing a biological sample into contact with an alkaline earth metal sulfate or an alkaline earth metal halide, heating the mixture as a pre-treatment, and then subjecting the pretreated product to an assay by use of a limulus reagent.

[0008] Patent Document 2 discloses an Et and BG assay method for blood samples, the method comprising mixing a biological sample with a hexadimethrine compound, an alkali metal hydroxide, a nonionic surfactant, and an alkaline earth metal halide, heating the mixture as a pre-treatment, and then subjecting the pre-treated product to an assay by use of a limulus reagent.

[0009] Patent Document 3 discloses an Et assay method for a sample possibly containing AT III, the method comprising bringing a biological sample into contact with a carrier on which lipopolysaccharide-binding peptide and/or lipid A-binding

peptide is immobilized to thereby recover Et via absorption into the carrier, and then subjecting the recovered Et to an assay by use of a limulus reagent or the like.

[0010] However, none of the aforementioned documents discloses or suggests subjecting AT III to a protein inactivation treatment as a pre-treatment in the co-presence of a divalent metal salt for carrying out limulus test of AT III.

Prior Art Documents

Patent Documents

[0011]

Patent Document 1: WO 2006/080448

Patent Document 2: Japanese Patent Application (*kokai*) No. Hei 6-70796

Patent Document 3: Japanese Patent Application (*kokai*) No. 2010-243342

Non-Patent Documents

[0012]

Non-Patent Document 1: Endotoxin and Natural Immunity Research 15 -Rapidly Developing Natural Immunity Research-, Igakutosho-shuppan Ltd., p. 27 to 30, 2012

Non-Patent Document 2: Endotoxin Research 12 - New Development in Natural Immunity Research-, Igakutosho-shuppan Ltd., p. 93 to 97, 2009

Summary of the Invention

Problems to be Solved by the Invention

[0013] An object of the present invention is to provide means for reducing reaction interference observed when AT III is subjected to limulus test, whereby limulus test of AT III can be carried out at high accuracy. The term "reaction interference" referred to herein refers to any action which is caused by a factor other than target substances of limulus test (e.g., Et and BG) and which affects the results of limulus test. Thus, "reaction interference" encompasses, for example, all actions affecting the results of limulus test caused by AT III itself, a component such as an additive or an impurity which is co-present with AT III, a component such as a reagent other than a pre-treatment agent which is co-present in the pre-treatment, and/or a component contained in a limulus reagent; such as an action of insolubilization of any of these components itself, an action of promoting or inhibiting the reaction of a limulus reagent, and an action of changing the micelle structure of Et or the steric structure of BG to thereby modify the activity of Et or BG (reactivity to Factor C or Factor G contained in a limulus reagent).

Means for Solving the Problems

[0014] The present inventors have conducted extensive studies so as to attain the aforementioned object, and have found that reaction interference observed when AT III is subjected to limulus test can be reduced by subjecting AT III to a protein inactivation treatment such as a thermal treatment or an acid treatment in the co-presence of a divalent metal salt, whereby limulus test of AT III can be carried out at high accuracy. The present invention has been accomplished on the basis of this finding.

[0015] Accordingly, the present invention encompasses the following modes.

- [1] A pre-treatment agent for antithrombin III to be subjected to limulus test, which agent contains a divalent metal salt, and which agent is used for subjecting antithrombin III to a protein inactivation treatment in the co-presence of the pre-treatment agent, before antithrombin III is subjected to limulus test.
- [2] The above-described pre-treatment agent, wherein the divalent metal salt is one or more metal salts selected from the group consisting of a divalent metal chloride, a divalent metal acetate, and a divalent metal sulfate.
- [3] The above-described pre-treatment agent, wherein the divalent metal constituting the divalent metal salt is one or more metals selected from the group consisting of magnesium, calcium, manganese, and zinc.
- [4] The above-described pre-treatment agent, wherein the concentration of the divalent metal ion derived from the

divalent metal salt is 0.5 mM or higher when antithrombin III is co-present with the pre-treatment agent.

[5] The above-described pre-treatment agent, wherein a target substance of the limulus test is endotoxin.

[6] A limulus reagent kit for antithrombin III, the kit comprising the pre-treatment agent.

[7] A pre-treatment method for antithrombin III to be subjected to limulus test, the method comprising subjecting antithrombin III to a protein inactivation treatment in the co-presence of the pre-treatment agent.

[8] The above-described pre-treatment method, wherein the protein inactivation treatment is a thermal treatment or an acid treatment.

[9] The above-described pre-treatment method, wherein the thermal treatment is performed at a temperature higher than 50°C.

[10] The above-described pre-treatment method, wherein the acid employed in the acid treatment is hydrochloric acid.

[11] A method for measuring a target substance of limulus test contained in antithrombin III, the method comprising preliminarily treating antithrombin III through the above-described pre-treatment method, and subjecting the preliminarily treated antithrombin III to limulus test.

[12] A method for producing antithrombin III, the method comprising measuring the target substance of limulus test in antithrombin III through the above-described method.

Brief Description of the Drawings

[0016]

[Fig. 1] A graph showing test results of parallel line assay.

[Fig. 2] A graph showing test results of parallel line assay.

[Fig. 3] A graph showing test results of parallel line assay.

Modes for Carrying Out the Invention

(1) The pre-treatment agent of the present invention

[0017] The pre-treatment agent of the present invention is a pre-treatment agent for AT III to be subjected to limulus test, which agent contains a divalent metal salt. The pre-treatment agent of the present invention is used for subjecting antithrombin III to a protein inactivation treatment in the co-presence of the pre-treatment agent of the present invention, before antithrombin III is subjected to limulus test.

[0018] The term "limulus test" referred to herein refers to a test for detecting (measuring) a target substance by use of a limulus reagent. The target substance may also be referred to as "target substance of limulus test (limulus test target substance)." The limulus test target substance is not particularly limited so long as it is a substance detectable by limulus test. The target substance is preferably Et or BG, and more preferably Et.

[0019] The "AT III" referred to herein is not particularly limited so long as it is a specimen containing AT III. The "AT III" referred to herein may consist of AT III, or may further contain a component other than AT III. The form of AT III is also not particularly limited. For example, AT III may be in the form of liquid, or may be in the form of solid such as a lyophilized product, powder, granules, or tablets.

[0020] An example of AT III is an injection preparation of AT III. Specific examples of such injection preparations include Neuart (registered trademark; product of Benesis Co., Ltd.), Anthrobin (product of The Chemo-Sero-Therapeutic Research Institute), and Nonthron (registered trademark; product of Nihon Pharmaceutical Co., Ltd.). When such an AT III injection preparation is subjected to the pre-treatment method of the present invention and limulus test, undiluted solution thereof may be used as a specimen, a concentrated solution thereof may be used as a specimen, or a solution diluted at any dilution factor with an aqueous medium (e.g., water or buffer) may be used as a specimen. The term "undiluted solution" referred to herein refers to a solution containing AT III at a concentration of 50 Unit/mL, which is a common concentration when AT III is used as an injection preparation. The "undiluted solution" may be obtained by dissolving AT III in an aqueous medium, according to the method described in a package insert of corresponding AT III injection preparation. The term "concentrated solution" referred to herein refers to a solution containing AT III at a concentration higher than 50 Unit/mL. An example of the "concentrated solution" is an intermediate purified product obtained in a step of production of an AT III injection preparation.

[0021] The dilution factor when undiluted solution is diluted with an aqueous medium is not particularly limited so long as a limulus test target substance can be detected. For example, the dilution factor is preferably higher than 1 and lower than 64, more preferably higher than 1 and 16 or lower, still more preferably higher than 1 and 10 or lower, yet more preferably higher than 1 and 4 or lower, particularly preferably higher than 1 and 2.5 or lower, remarkably preferably 1.1 to 2.5, particularly remarkably preferably 1.2 to 2.5. Notably, the term "dilution factor" referred to herein refers to the dilution fold of a reaction mixture in which a protein inactivation treatment is performed in the co-presence of the pre-

treatment agent of the present invention, with respect to the aforementioned "undiluted solution." Thus, a dilution factor of "1" refers to the state that the AT III concentration of a reaction mixture in which a protein inactivation treatment is performed in the co-presence of the pre-treatment agent of the present invention is the same as the AT III concentration of the aforementioned "undiluted solution." In other words, a dilution factor of "1" refers to the state that the reaction mixture is the same as the undiluted solution, and also the state that the reaction mixture is not diluted and thereby contains AT III at a concentration of 50 Unit/mL.

[0022] AT III may also be a recombinant protein. The recombinant AT III may be obtained through gene expression in a host cell. Expression in a host cell may be performed through a conventional method. For example, expression in a host cell may be performed through a method by Yamada *et al.* (WO 2005/035563). The amino acid sequence of AT III and the nucleotide sequence of a gene encoding AT III may be obtained from a known database. Examples of such a known database include NCBI (<http://www.ncbi.nlm.nih.gov/>). The recombinant protein to be expressed may be a protein having an amino acid sequence identical to that of a native AT III. In addition, the recombinant protein to be expressed may be a variant of a native AT III, so long as functions of AT III are maintained. Such a variant encompasses homologues and artificially modified products of known AT III.

[0023] The "divalent metal salt" referred to herein is not particularly limited so long as it is a metal salt recognized as a divalent metal salt in the art of the present invention. Examples of the divalent metal salt include a divalent metal fluoride, a divalent metal chloride, a divalent metal bromide, a divalent metal iodide, a divalent metal hydroxide, a divalent metal cyanide, a divalent metal nitrate, a divalent metal nitrite, a divalent metal hypochlorite, a divalent metal chlorite, a divalent metal chlorate, a divalent metal perchlorate, a divalent metal permanganate, a divalent metal acetate, a divalent metal hydrogencarbonate, a divalent metal dihydrogen phosphate, a divalent metal hydrogensulfate, a divalent metal hydrosulfide salt, a divalent metal thiocyanate, a divalent metal oxide, a divalent metal sulfide, a divalent metal peroxide, a divalent metal sulfate, a divalent metal sulfite, a divalent metal thiosulfate, a divalent metal carbonate, a divalent metal chromate, a divalent metal bichromate, a divalent metal monohydrogen phosphate, and a divalent metal phosphate. As the divalent metal salt, a single kind of metal salt may be used solely, or two or more kinds of metal salts may be used in combination. The divalent metal salt is preferably, for example, one or more metal salts selected from the group consisting of a divalent metal chloride, a divalent metal acetate, and a divalent metal sulfate.

[0024] The divalent metal salt may be a metal salt which provides a divalent metal ion when AT III is co-present with the pre-treatment agent of the present invention. The state "when AT III is co-present with the pre-treatment agent of the present invention" specifically refers to the duration in which the protein inactivation treatment mentioned below is performed. The divalent metal salt may be, for example, a metal salt which provides a divalent metal ion through dissolution in an aqueous medium. Examples of the aqueous medium include water and a buffer.

[0025] The "divalent metal" referred to herein is not particularly limited so long as it is a metal recognized as a divalent metal in the art of the present invention. The "divalent metal" may refer to a metal which provides a divalent metal ion when ionized. Examples of the divalent metal include beryllium, magnesium, calcium, strontium, barium, radium, cadmium, nickel, zinc, copper, mercury, iron, cobalt, tin, lead, and manganese. As the divalent metal, a single kind of metal may be used solely, or two or more kinds of metals may be used in combination. The divalent metal is preferably, for example, one or more metals selected from the group consisting of magnesium, calcium, manganese, and zinc.

[0026] Specific examples of the divalent metal salt include magnesium sulfate (MgSO_4), magnesium chloride (MgCl_2), magnesium acetate ($\text{Mg}(\text{CH}_3\text{COO})_2$), calcium chloride (CaCl_2), calcium acetate ($\text{Ca}(\text{CH}_3\text{COO})_2$), manganese sulfate (MnSO_4), and zinc sulfate (ZnSO_4). The pre-treatment agent of the present invention may contain, for example, only magnesium sulfate (MgSO_4) as a divalent metal salt. Alternatively, the pre-treatment agent of the present invention may contain, for example, magnesium sulfate (MgSO_4) and one or more other divalent metal salts.

[0027] Also, the pre-treatment agent of the present invention may further contain an additional component. The "additional component" is not particularly limited so long as it is acceptable for use in a reagent or diagnostic agent. For example, components which are incorporated into a reagent or a diagnostic agent may be employed as the "additional component". Examples of the "additional component" include an alkali metal chloride, an alkali metal acetate, an alkali metal sulfate, a surfactant, and an additive. Examples of the additive include, but are not limited to, a stabilizer, an emulsifying agent, an osmo-regulating agent, a buffer, a tonic agent, a preservative, a pH-adjusting agent, a colorant, an excipient, a condensing agent, a lubricant, and a disintegrant.

[0028] The form of the pre-treatment agent of the present invention is not particularly limited. For example, the pre-treatment agent of the present invention may be provided as liquid preparation, or may be provided as solid preparation for dissolution upon use; such as powder, granules, or tablets, may be employed. Also, the pre-treatment agent of the present invention may be provided as a microplate retaining the pre-treatment agent of the present invention in the wells thereof. Such a microplate may be obtained through, for example, dispensing the pre-treatment agent of the present invention which has been prepared in the form of liquid into the wells and drying the plate. In the case where the pre-treatment agent of the present invention is provided as a liquid preparation, the pre-treatment agent of the present invention may be provided as a frozen product, or may be provided as a liquid product (melting state).

[0029] In the pre-treatment agent of the present invention, the divalent metal salt may be present as a salt form, an

ionic form, or a mixture thereof. The state that "the pre-treatment agent of the present invention contains a divalent metal salt" encompasses all three cases.

[0030] Except for incorporation of a divalent metal salt, the pre-treatment agent of the present invention may be produced through the same technique as generally employed in production of reagents and diagnostic agents.

[0031] The concentration of the divalent metal salt concentration in the pre-treatment agent of the present invention is not particularly limited, and the concentration may be appropriately tuned in accordance with various conditions such as the type of the divalent metal salt and the amount used of the pre-treatment agent of the present invention. The concentration of the divalent metal salt in the pre-treatment agent of the present invention may be, for example, 0.001 mass% or higher, 0.01 mass% or higher, 0.1 mass% or higher, 1 mass% or higher, 5 mass% or higher, 10 mass% or higher, 30 mass% or higher, or 50 mass% or higher. The concentration of the divalent metal salt in the pre-treatment agent of the present invention may be, for example, 100 mass% or lower, 50 mass% or lower, 30 mass% or lower, 10 mass% or lower, 5 mass% or lower, or 1 mass% or lower.

[0032] The amount used of the pre-treatment agent of the present invention is not particularly limited, and the amount may be appropriately tuned in accordance with various conditions such as the type and the concentration of the divalent metal salt. The pre-treatment agent of the present invention may be used so that, for example, the concentration of the divalent metal ion derived from the divalent metal salt (hereinafter the concentration may be referred to as "divalent metal ion concentration during treatment") falls within the below-exemplified ranges when AT III is co-present with the pre-treatment agent of the present invention. The "concentration when AT III is co-present with the pre-treatment agent of the present invention" specifically refers to the concentration of the divalent metal ion in the reaction system where the protein inactivation treatment mentioned below is performed. The divalent metal ion concentration during treatment is not particularly limited so long as the effect of the pre-treatment of the present invention can be attained, and the concentration is preferably, for example, 0.5 mM or higher. The divalent metal ion concentration during treatment is, for example, preferably 0.5 mM to 100 mM, more preferably 0.5 mM to 50 mM, still more preferably 0.5 mM to 25 mM, yet more preferably 5 mM to 25 mM, particularly preferably 5 mM to 12.5 mM. Notably, in the case where the pre-treatment agent of the present invention contains two or more divalent metal salts, the "divalent metal ion concentration during treatment" means the total concentration of the divalent metal ion(s) derived from these divalent metal salts.

[0033] Through a pre-treatment of AT III by use of the pre-treatment agent of the present invention, reaction interference observed when AT III is subjected to limulus test can be reduced, and thus, limulus test of AT III can be carried out at high accuracy.

(2) The kit of the present invention

[0034] The kit of the present invention is a limulus reagent kit which comprises the pre-treatment agent of the present invention, as a component. The kit of the present invention may further comprise an additional component. Examples of the "additional component" include, but are not limited to, a limulus reagent, a substrate for detection of limulus reaction, buffer, distilled water, a standard substance (e.g., Et or BG), and a microplate.

[0035] The limulus reagent used in the present invention is not particularly limited so long as the reagent contains a protein or proteins involved in a cascade reaction corresponding to a limulus test target substance. An example of the limulus reagent is blood corpuscle extracts of horseshoe crab (LAL; lysate reagent). LAL may be obtained from blood of a horseshoe crab as a raw material through a conventional method. LAL may be used after appropriate fractionation and/or purification. Any types of horseshoe crabs may be employed as the origin of LAL. Example of the horseshoe crab include *Tachypleus tridentatus* (Japanese horseshoe crab), *Limulus polyphemus* (American horseshoe crab), *Carciniscorpius rotundicauda* (Southeast Asian horseshoe crab), and *Tachypleus gigas* (Southeast Asian horseshoe crab). Examples of the limulus reagent employing a lysate derived from *Limulus polyphemus* include Endospecy (registered trademark) ES-50M (product of Seikagaku Corporation), Pyrochrome (product of Associates of Cape Cod, Inc.), Pyrotell (registered trademark)-T (product of Associates of Cape Cod, Inc.), Pyrotell (registered trademark) multi-test (product of Associates of Cape Cod, Inc.), Kinetic-QCL (product of Lonza Walkersville, Inc.) and Endochrome-K (product of Charles River Laboratories, Inc.).

[0036] Examples of the limulus reagent also include artificially reconstituted limulus reagents. The reconstituted limulus reagent is not particularly limited so long as the reagent contains a protein or proteins involved in a cascade reaction corresponding to a limulus test target substance. Proteins involved in the cascade reaction are also collectively referred to as "factors." The term "cascade reaction" referred to herein refers to a series of reactions which proceed due to the presence of a limulus test target substance. For example, when a substance which activates Factor C (e.g., Et) is present, a series of the following reactions proceed: Factor C is activated by the substance to thereby form activated Factor C; the activated factor C activates Factor B to thereby form activated Factor B; and the activated Factor B activates Pro-clotting enzyme to thereby form the corresponding Clotting enzyme. That is, in the case where a substance which activates Factor C (e.g., Et) is a target substance, it is sufficient that the reconstituted limulus reagent contains Factor C, Factor B, and Pro-clotting enzyme, as factors constituting the cascade reaction. When a substance which activates

Factor G (e.g., BG) is present, a series of the following reactions proceed: Factor G is activated by the substance to thereby form activated Factor G; and the activated Factor G activates Pro-clotting enzyme to thereby form the corresponding Clotting enzyme. That is, in the case where a substance which activates Factor G (e.g., BG) is a target substance, it is sufficient that the reconstituted limulus reagent contains Factor G and Pro-clotting enzyme, as factors constituting the cascade reaction. The progress of a cascade reaction can be determined by detecting Clotting enzyme by use of the below-mentioned detection substrate for limulus reaction.

[0037] In the case where a detection substrate that is able to detect an intermediate stage of a cascade reaction is employed as the below-mentioned detection substrate for limulus reaction, it is sufficient that the reconstituted limulus reagent contains a factor or factors involved in from the start of the cascade reaction to the relevant intermediate stage. Specifically, for example, in the case where a substance which activates Factor C (e.g., Et) is a target substance, and a detection substrate for limulus reaction that is able to detect activated Factor C is used, it is sufficient that the reconstituted limulus reagent contains Factor C.

[0038] Each of factors contained in a reconstituted limulus reagent may be a native protein or a recombinant protein.

[0039] Native factors may each be obtained from amebocyte lysates (LAL) of the aforementioned various horseshoe crabs. These factors may each be purified to a desired degree before use. Purification may be performed through, for example, a known technique (Nakamura T. et al., J. Biochem. 1986 Mar; 99(3): 847-57).

[0040] The factors may each also be obtained through expression in a host cell. Expression in a host cell may be performed through a conventional method. For example, expression in a host cell may be performed through a method by Mizumura et al., (WO 2012/118226). The amino acid sequence of each factor and the nucleotide sequence of a gene encoding the factor may be obtained from a known database. Examples of such a known database include NCBI (<http://www.ncbi.nlm.nih.gov/>). The thus-expressed recombinant proteins may each be purified to a desired degree before use, as necessary.

[0041] By appropriately combining the factors, a reconstituted limulus reagent can be obtained. The factors contained in the reconstituted limulus reagent may be all native factors, all recombinant proteins, or any combination thereof. Also, the reconstituted limulus reagent may be an appropriate combination of the factor or factors with a whole LAL, or with LAL which has been subjected to appropriate fractionation and/or purification or the like.

[0042] The detection substrate for limulus reaction (hereinafter may also be referred to simply as "detection substrate") refers to a substrate for detecting the progress of the cascade reaction.

[0043] Examples of the detection substrate include coagulogen. Coagulogen is a detection substrate with respect to Clotting enzyme, which is the final product of the cascade reaction. When coagulogen come into contact with Clotting enzyme, a coagulated product, coagulin, is formed. The progress of the coagulation reaction can be determined by measuring the turbidity of the reaction mixture or by observing gel formation. Coagulogen can be recovered from blood corpuscle extracts of horseshoe crab (LAL). Since the nucleotide sequence of the gene encoding coagulogen has been determined (Miyata et al, Protein Nucleic acid Enzyme, extra issue, No. 29; p. 30-43; 1986), coagulogen can also be produced genetically according to a conventional technique.

[0044] The detection substrate may be a synthetic substrate. The synthetic substrate is not particularly limited so long as it has a property suitable for detection of the progress of the cascade reaction. The "property suitable for detection of the progress of the cascade reaction" may be, for example, a property for detecting the presence of Clotting enzyme or a property for detecting an intermediate stage of the cascade reaction. Examples of the "property for detecting the presence of Clotting enzyme" include a property of coloring by an enzymatic reaction of Clotting enzyme and a property of generating fluorescence by an enzymatic reaction of Clotting enzyme. Examples of the "property for detecting an intermediate stage of the cascade reaction" include a property of coloring by an enzymatic reaction of activated Factor C or the like, and a property of generating fluorescence by an enzymatic reaction of activated Factor C or the like. Examples of the synthetic substrate include a substrate represented by formula X-Y-Z (wherein X represents a protection group, Y represents a peptide, and Z represents a dye linked to Y via amide bond). When a substance which activates Factor C (e.g., Et) is present in the reaction system containing Factor C, Factor B, and Pro-clotting enzyme, an amide linkage Y-Z is cleaved by the enzymatic reaction of Clotting enzyme, which is yielded by the cascade reaction, to release the dye Z, whereby coloring occurs or fluorescence is generated. In the case where the reaction system containing Factor G and Pro-clotting enzyme is employed, or in the case where an intermediate stage of the cascade reaction is detected, it is sufficient that the dye Z is released by Clotting enzyme or by a protein formed yielded at the intermediate stage of the cascade reaction, respectively. The protective group X is not particularly limited, and a known protective group for peptide may be suitably used. Examples of such a protective group include a t-butoxycarbonyl group and a benzoyl group. The dye Z is not particularly limited, and it may be a dye detectable under visible light or may be a fluorescent dye. Examples of the dye Z include p-nitroaniline (pNA), 7-methoxycoumarin-4-acetic acid (MCA), -2,4-dinitroaniline (DNP), and Dansyl dyes. Examples of the peptide Y include Leu-Gly-Arg (LGR), Ile-Glu-Gly-Arg (IEGR) (SEQ ID NO: 1), Val-Pro-Arg (VPR), and Asp-Pro-Arg (DPR). These synthetic substrates may each be used for detecting the presence of Clotting enzyme, or for detecting an intermediate stage of the cascade reaction. Among these synthetic substrates, suitable one may be appropriately selected and used depending on the detection target. For example, from

the viewpoint of substrate specificity, a substrate comprising LGR as peptide Y can be suitably used for detecting Clotting enzyme, and a substrate comprising VPR or DPR as peptide Y can be suitably used for detecting activated Factor C. The released dye Z may be determined through a technique in accordance with the property of the dye.

[0045] When blood corpuscle extracts of horseshoe crab (LAL) is used as a limulus reagent, coagulogen, which is intrinsically contained in LAL, may be used as a detection substrate. Also, an appropriately selected detection substrate may be used in combination with LAL.

[0046] When a reconstituted limulus reagent is used as a limulus reagent, an appropriately selected detection substrate may be combined with the reconstituted limulus reagent.

[0047] In the kit of the present invention, these factors and detection substrates may be present as a mixture thereof, as separate components, or as separate arbitrary combinations thereof.

[0048] Through the pre-treatment of AT III with the kit of the present invention, reaction interference observed when AT III is subjected to limulus test can be reduced, whereby limulus test of AT III can be carried out at high accuracy.

(3) The pre-treatment method of the present invention

[0049] The pre-treatment method of the present invention is a method for preliminarily treating AT III to be subjected to limulus test, which method comprises subjecting AT III to a protein inactivation treatment in the co-presence of the pre-treatment agent of the present invention.

[0050] The "protein inactivation treatment" referred to herein is not particularly limited so long as it is a treatment of inactivating AT III, or more specifically a treatment of inactivating AT III to such an extent that limulus test is not inhibited. Examples of the protein inactivation treatment include, but are not limited to, a thermal treatment, an acid treatment, and an alkali treatment. As the protein inactivation treatment, a single kind of treatment may be used solely, or two or more kinds of treatments may be used in combination.

[0051] The protein inactivation treatment may be performed in, for example, an aqueous medium such as water or a buffer. That is, the protein inactivation treatment may be performed in, for example, an aqueous medium containing AT III in the co-presence of the pre-treatment agent of the present invention.

[0052] The term "thermal treatment" referred to herein refers to heating of AT III in the co-presence of the pre-treatment agent of the present invention. The method for heating is not particularly limited, and a method using a heating apparatus such as a heat block, a water bath, an air bath, or a microwave oven is preferably employed. The temperature of the thermal treatment is not particularly limited so long as protein inactivation occurs. For example, the temperature of the thermal treatment is generally higher than 50°C. The temperature of the thermal treatment is preferably, for example, 55°C to 100°C, more preferably 55°C to 95°C, still more preferably 60°C to 90°C, yet more preferably 65°C to 85°C, particularly preferably 65°C to 75°C. The time of the thermal treatment is not particularly limited so long as protein inactivation occurs. For example, the time of the thermal treatment is preferably 1 minute or longer. The time of the thermal treatment is, for example, preferably 1 minute to 2 hours, more preferably 1 minute to 1 hour, still more preferably 5 minutes to 30 minutes, yet more preferably 5 minutes to 20 minutes, particularly preferably 5 minutes to 15 minutes.

[0053] The term "acid treatment" referred to herein refers to a treatment of bringing AT III co-present with the pre-treatment agent of the present invention into contact with an acid. Examples of the acid used in the acid treatment include, but are not limited to, hydrochloric acid, sulfuric acid, and nitric acid. Of these, hydrochloric acid is preferred. The amount of acid added may be appropriately tuned in accordance with various conditions such as the type of acid. The amount of acid added may be, for example, such an amount that the acid concentration of the reaction system at the acid treatment becomes 0.001 N to 1 N, preferably 0.01 N to 0.5 N, more preferably 0.01 N to 0.1 N. The time of acid treatment is not particularly limited so long as protein inactivation occurs, and it may be a time generally employed in an acid treatment. The time of acid treatment may be, for example, 0.1 seconds to 2 hours.

[0054] The term "alkali treatment" referred to herein refers to a treatment of bringing AT III co-present with the pre-treatment agent of the present invention into contact with an alkali. Examples of the alkali used in the alkali treatment include, but are not limited to, sodium hydroxide and potassium hydroxide. Of these, sodium hydroxide is preferred. The amount of alkali added may be appropriately tuned in accordance with various conditions such as the type of alkali. The amount of alkali added may be, for example, such an amount that the alkali concentration of the reaction system at the alkali treatment becomes 0.001 N to 1 N, preferably 0.01 N to 0.5 N, more preferably 0.01 N to 0.1 N. The time of alkali treatment is not particularly limited so long as protein inactivation occurs, and it may be a time generally employed in an alkali treatment. The time of alkali treatment may be, for example, 0.1 seconds to 2 hours.

[0055] The pre-treatment method of the present invention may further include a step other than the protein inactivation treatment.

[0056] For example, an additional treatment may be appropriately performed on AT III before the protein inactivation treatment. For example, a processing treatment of AT III, such as particle size reduction of AT III dry powder, may be performed. Also, AT III may be diluted or concentrated. These additional treatments may each be performed before AT III is made to be co-present with the pre-treatment agent of the present invention, or after AT III is made to be co-present

with the pre-treatment agent of the present invention and before the protein inactivation treatment.

[0057] Also, after the protein inactivation treatment, a post treatment may be appropriately performed. The post treatment may be, for example, a treatment of preventing inhibition of limulus test when preliminarily treated AT III is subjected to limulus test. For example, when a thermal treatment has been performed as a protein inactivation treatment, the thus-treated product may be cooled. Also, for example, when an acid treatment is performed as a protein inactivation treatment, the thus-treated product may be neutralized with alkali. Also, for example, when an alkali treatment is performed as a protein inactivation treatment, the thus-treated product may be neutralized with acid. Also, for example, such a treated product may be diluted or concentrated.

[0058] Through the pre-treatment of AT III by the pre-treatment method of the present invention, reaction interference observed when AT III is subjected to limulus test can be reduced, whereby limulus test of AT III can be carried out at high accuracy.

(4) Assay method of the present invention

[0059] The assay method of the present invention is a method for measuring a limulus test target substance contained in AT III, the method comprising preliminarily treating AT III through the pre-treatment method of the present invention, and subjecting the preliminarily treated AT III to limulus test.

[0060] Limulus test may be carried out through, for example, a known technique. Examples of such a known technique include chromogenic assay, turbidimetric assay, and gel-clot assay.

[0061] Specifically, limulus test can be carried out by bringing AT III into contact with a limulus reagent. When a limulus test target substance is present in AT III, a cascade reaction proceeds. Thus, the limulus test target substance in AT III can be measured by determining the progress of the cascade reaction. In limulus test, the factors may be contained in the reaction system at the start of the step of bringing AT III into contact with the limulus reagent, or may be successively added to the reaction system.

[0062] The progress of the cascade reaction can be determined by use of a detection substrate. That is, the progress of the cascade reaction can be determined by measuring the response (coloring, coagulation, etc.) of the detection substrate depending on the type of the detection substrate. The detection substrate may be added to the reaction system in any timing. For example, the detection substrate may be contained in the reaction system at the start of the step of bringing AT III into contact with a limulus reagent, or may be added during or after the step. In the case where a limulus reagent containing a detection substrate is used, no additional detection substrate needs to be added to the reaction system.

[0063] The assay method of the present invention may further comprise any additional step, so long as a cascade reaction proceeds when AT III contains a limulus test target substance. For example, as described above, the assay method of the present invention may further comprise a step of adding a detection substrate to the reaction system. Also, the assay method of the present invention may further comprise a step of transforming data obtained through measurement into different data. An example of the step of transforming data obtained through measurement into different data is a step of calculating the amount of a limulus test target substance present in AT III on the basis of the data obtained through measurement.

[0064] In the assay method of the present invention, any reaction is preferably performed in an aqueous medium such as water or a buffer.

[0065] According to the assay method of the present invention, reaction interference observed when AT III is subjected to limulus test can be reduced, whereby limulus test of AT III can be carried out at high accuracy.

[0066] Thus, there can be obtained AT III which has been confirmed to be free from contamination of a limulus test target substance. That is, the present invention also provides a method for producing AT III, the method comprising measuring a limulus test target substance in AT III through the assay method of the present invention. The thus-produced AT III may be provided in any form, such as an injection preparation.

Examples

[0067] The present invention will next be described more specifically by way of examples, which should not be construed as limiting the technical scope of the present invention. Notably, water, reagents, plastic articles, glass articles, and others used in all tests described in the Examples were ensured to contain no endotoxin and no reaction interference factor with respect to the reaction of a limulus reagent.

<Definition of Et recovery>

[0068] In tests carried out through chromogenic assay or turbidimetric assay, the mean recovery of the added Et (Et recovery) was calculated through the following procedure. Specifically, as well as limulus test for an AT III specimen

spiked with a known amount of Et, limulus test for a specimen employing an equivolume of water instead of AT III and spiked with Et in the same way was concurrently carried out as a positive control. The absorbance change rate (mAbs/min) of the AT III specimen was divided by the absorbance change rate of the positive control, and thus-obtained value was converted to a percentage, to thereby obtain the Et recovery. In the case that AT III or water is contaminated with Et, the Et recovery may be calculated after subtraction of a contamination-equivalent value from a corresponding measurement. That is, the concept "Et recovery" may refer to the ratio (%) of the amount of Et actually detected in an AT III specimen to the amount of Et to be theoretically detected in the case where reaction interference by AT III is absent.

<Referential Example 1>

Et recovery test for AT III, investigated through dilution and heating method

[0069] An undiluted 50-Unit/mL AT III preparation (Neuart (registered trademark; product of Benesis; hereinafter the same shall apply), and water-diluted preparations thereof at dilution factors of 4, 16, 64, and 256 were provided. To each of the undiluted and diluted preparations (0.4 mL), a standard solution (0.04 mL) of Et (JPRSE: Japanese Pharmacopoeia endotoxin standard sample; hereinafter the same shall apply), prepared to a concentration of 0.5 EU/mL, was added. The thus-prepared mixtures were each vigorously agitated for one minute by means of a test tube mixer. Subsequently, each solution (0.2 mL) was dispensed to another container, and heated at 70°C for 10 minutes by means of a heat block. Thereafter, the thus-heated solution (0.05 mL) was dispensed to a microplate, and an Et assay reagent for chromogenic assay (Endospecy (registered trademark) ES-50M; product of Seikagaku Corporation; hereinafter the same shall apply) (0.05 mL) was added to the microplate. The microplate was vigorously agitated for one minute by means of a well-reader provided with functions of a thermostat bath and agitation (Well-reader MP-96; product of Seikagaku Corporation; hereinafter the same shall apply). Thereafter, the change over time in absorbance was monitored at a measurement wavelength of 405 nm and a reference wavelength of 492 nm, while the microplate was heated at 37°C for 30 minutes, and then the Et recovery was calculated. Table 1 shows the results.

[Table 1]

Table 1: Results of Et detection in AT III through dilution and heating method		
	Dilution factor	Et recovery
	× 1	7.3 %
	× 4	5.8 %
	× 16	9.4 %
	× 64	120.0 %
	× 256	100.3 %

[0070] As shown in Table 1, when the dilution factor was 16 or lower, the Et recovery was lower than 10%, failing to detect Et at high accuracy. In contrast, when the dilution factor was 64 or higher, the Et recovery of about 100% was attained. The results were virtually the same as previously reported results of the dilution heating method (Non-Patent Document 1).

<Example 1>

Et recovery test, wherein thermal treatment was performed in the presence of magnesium sulfate or calcium chloride

[0071] A 0.5-EU/mL Et standard solution (0.1 mL) was added to a 25-Unit/mL AT III preparation (AT III preparation twice diluted with water) (0.9 mL), and the mixture was vigorously agitated for one minute by means of a test tube mixer. Subsequently, the mixture (0.18 mL) was dispensed to another container, and 15.6 mM, 125 mM, 250 mM, 500 mM, or 1,000 mM aqueous MgSO₄ (0.02 mL), or 15.6 mM, 56 mM, 125 mM, 250 mM, or 500 mM aqueous CaCl₂ (0.02 mL) was added thereto, followed by vigorously agitating for one minute by means of a test tube mixer. Each solution was heated for 10 minutes at 70°C by means of a heat block, and the solution was immediately cooled with ice. The solution was returned to room temperature, and an aliquot (0.05 mL) thereof was dispensed to a microplate. Endospecy ES-50 M (0.05 mL) was added to the microplate, and the microplate was vigorously agitated for one minute by means of a well-reader. Thereafter, the change over time in absorbance was monitored at a measurement wavelength of 405 nm and a reference wavelength of 492 nm, while the microplate was heated at 37°C for 30 minutes, and then the Et recovery was calculated. Tables 2 and 3 show the results. In the tables, "MgSO₄ concentration" and "CaCl₂ concentration" are

those of each reaction mixture during the thermal treatment. The dilution factor of the AT III preparation employed in Example 1 was about 2.5.

[Table 2]

Table 2: Results of Et detection in the case of thermal treatment in the co-presence of magnesium sulfate

MgSO ₄ concentration	Et recovery
1.56 mM	0.0 %
12.5 mM	95.0 %
25 mM	108.0 %
50 mM	99.9 %
100 mM	93.7 %

[Table 3]

Table 3: Results of Et detection in the case of thermal treatment in the co-presence of calcium chloride

CaCl ₂ concentration	Et recovery
1.56 mM	6.4 %
5.6 mM	75.0 %
12.5 mM	52.4 %
25 mM	48.0 %
50 mM	13.1 %

[0072] As shown in Tables 2 and 3, in contrast to the case of the dilution and heating method, when AT III and Et were subjected to a thermal treatment in the co-presence of magnesium sulfate or calcium chloride, Et could be detected at high accuracy without being diluted at a dilution factor of 64 or higher. Particularly, when the magnesium sulfate concentration was 12.5 mM or higher, Et could be detected at high accuracy.

<Example 2>

Investigation into type of divalent metal salts

[0073] A 0.5-EU/mL Et standard solution (0.22 mL) was added to a 50-Unit/mL AT III preparation (0.2 mL), and the mixture was vigorously agitated for one minute by means of a test tube mixer. Subsequently, water (1.8 mL) was added thereto, and the mixture was vigorously agitated for one minute by means of a test tube mixer. An aliquot (0.1 mL) of the mixture was dispensed another container. A 50 mM or 5 mM aqueous solution of a divalent metal salt was added in a 1/10 amount (0.011 mL) thereto, and the mixture was vigorously agitated for one minute by means of a test tube mixer. Each solution was heated for 10 minutes at 70°C by means of a heat block, and the solution was immediately cooled with ice. The solution was returned to room temperature, and an aliquot (0.05 mL) thereof was dispensed to a microplate. Endospecy ES-50 M (0.05 mL) was added to the microplate, and the microplate was vigorously agitated for one minute by means of a well-reader. Thereafter, the change over time in absorbance was monitored at a measurement wavelength of 405 nm and a reference wavelength of 492 nm, while the microplate was heated at 37°C for 30 minutes, and then the Et recovery was calculated. Table 4 shows the results. In the table, the divalent metal salt concentration is that of the reaction mixture during the thermal treatment. The dilution factor of the AT III preparation employed in Example 2 was about 12.

[Table 4]

Table 4: Results of Et detection in the case of thermal treatment in the co-presence of a divalent metal salt

Co-present divalent metal salt	Et recovery
0.5 mM Mg (CH ₃ COO) ₂	56.8 %
5.0 mM Mg (CH ₃ COO) ₂	104.7 %
0.5 mM MgCl ₂	11.1 %

(continued)

Table 4: Results of Et detection in the case of thermal treatment in the co-presence of a divalent metal salt

Co-present divalent metal salt	Et recovery
5.0 mM MgCl ₂	106.2 %
0.5 mM Ca (CH ₃ COO) ₂	75.7 %
5.0 mM Ca (CH ₃ COO) ₂	84.6 %
0.5 mM MnSO ₄	110.7 %
5.0 mM MnSO ₄	88.1 %
0.5 mM ZnSO ₄	105.7 %
5.0 mM ZnSO ₄	15.7 %

[0074] As shown in Table 4, Et could be detected in the case where AT III was subjected to a thermal treatment in the co-presence of Mg(CH₃COO)₂, MgCl₂, Ca(CH₃COO)₂, MnSO₄, or ZnSO₄.

<Example 3>

Investigation into temperature and time of thermal treatment

[0075] A 0.5-EU/mL Et standard solution (0.1 mL) was added to a 50-Unit/mL AT III preparation (0.9 mL), and the mixture was vigorously agitated for one minute by means of a test tube mixer. Subsequently, an aliquot (0.18 mL) of the mixture was dispensed another container. Subsequently, 200 mM aqueous CaCl₂ (0.02 mL) was added thereto, and the mixture was vigorously agitated for one minute by means of a test tube mixer. Each solution was placed on ice or heated at 37°C, 50°C, 60°C, 70°C, 80°C, or 90°C for 5 minutes, 10 minutes, or 20 minutes by means of a heat block. In the case of heating, the solution was cooled with ice immediately after completion of heating. Each solution was returned to room temperature, and an aliquot (0.05 mL) thereof was dispensed to a microplate. Endospecy ES-50 M (0.05 mL) was added to the microplate, and the microplate was vigorously agitated for one minute by means of a well-reader. Thereafter, the change over time in absorbance was monitored at a measurement wavelength of 405 nm and a reference wavelength of 492 nm, while the microplate was heated at 37°C for 30 minutes, and then the Et recovery was calculated. Table 5 shows the results. The CaCl₂ concentration of the reaction mixture during the thermal treatment was 20 mM. The dilution factor of the AT III preparation employed in Example 3 was about 1.2.

[Table 5]

Table 5: Results of Et detection in the case of thermal treatment under various conditions

Temp.	Time	Et recovery
0°C	10 min.	0.0 %
37°C	5 min.	0.0 %
	10 min.	0.0 %
50°C	10 min.	0.0 %
60°C	5 min.	35.7 %
	10 min.	46.3 %
70°C	5 min.	52.3 %
	10 min.	61.9 %
	20 min.	68.6 %
80°C	10 min.	57.9 %
90°C	10 min.	56.5 %

[0076] As shown in Table 5, when the thermal treatment was performed at 60°C or higher, Et could be detected. Also, when the thermal treatment was performed for 5 minutes or longer, Et could be detected. Based on the results obtained for heating at the tested temperature (60°C, 70°C, 80°C, or 90°C) for 10 minutes, a thermal treatment temperature of

70°C, at which the highest Et recovery was obtained, was conceived to be preferred.

<Referential Example 2>

Investigation into effect of addition of divalent metal salt after thermal treatment

[0077] A 0.5-EU/mL Et standard solution (0.1 mL) was added to water (0.9 mL) or to a 4-fold or 16-fold water-diluted 50-Unit/mL AT III preparation (0.9 mL), and the mixture was vigorously agitated for one minute by means of a test tube mixer. Subsequently, an aliquot (0.18 mL) of the mixture was dispensed another container, and each mixture was placed on ice or heated at 70°C for 10 minutes by means of a heat block. In the case of heating, the solution was cooled with ice immediately after completion of heating. Each solution was returned to room temperature. Thereafter, water or 7 mM, 28 mM, or 112 mM aqueous CaCl_2 (0.02 mL) was added thereto, and the mixture was vigorously agitated for one minute by means of a test tube mixer. An aliquot (0.05 mL) thereof was dispensed to a microplate. Endospecy ES-50 M (0.05 mL) was added to the microplate, and the microplate was vigorously agitated for one minute by means of a well-reader. Thereafter, the change over time in absorbance was monitored at a measurement wavelength of 405 nm and a reference wavelength of 492 nm, while the microplate was heated at 37°C for 30 minutes, and then the Et recovery was calculated. Table 6 shows the results.

[Table 6]

Table 6: Results of Et detection in the case of adding divalent metal salt after thermal treatment			
	Dilution factor	CaCl_2 concentration	Et recovery
x 4		0 mM	8.0 %
		0.7 mM	6.9 %
		2.8 mM	6.3 %
		11.2 mM	5.9 %
x 16		0 mM	9.6 %
		0.7 mM	6.0 %
		2.8 mM	5.7 %
		11.2 mM	5.5 %

[0078] As shown in Table 6, in the method employing addition of a divalent metal salt after the thermal treatment, Et recovery was lower than 10%, failing to detect Et at high accuracy. Thus, it was elucidated that the divalent metal salt must be co-present with AT III and Et before the thermal treatment.

<Example 4>

Investigation into acid treatment

[0079] A 0.5-EU/mL Et standard solution (0.22 mL) was added to a 50-Unit/mL AT III preparation (0.2 mL), and the mixture was vigorously agitated for one minute by means of a test tube mixer. Subsequently, water (1.8 mL) was added thereto, and the mixture was vigorously agitated for one minute by means of a test tube mixer. Subsequently, an aliquot (0.1 mL) of the mixture was dispensed another container. A 50 mM or 5 mM aqueous solution of a divalent metal salt was added in a 1/10 amount (0.011 mL) thereto, and the mixture was vigorously agitated for one minute by means of a test tube mixer. Thereafter, 5N, 2N, 1N, 0.5N, or 0.1N HCl (0.011 mL) was added thereto, and the mixture was vigorously agitated for one minute by means of a test tube mixer. An aliquot (0.05 mL) thereof was dispensed to a microplate. Endospecy ES-50 M (0.05 mL) was added to the microplate, and the microplate was vigorously agitated for one minute by means of a well-reader. Thereafter, the change over time in absorbance was monitored at a measurement wavelength of 405 nm and a reference wavelength of 492 nm, while the microplate was heated at 37°C for 30 minutes, whereby and then the Et recovery was calculated. Table 7 shows the results. In Table 7, the divalent metal salt concentration and hydrochloric acid concentration are those of the reaction mixtures during the acid treatment (estimated values). The dilution factor of the AT III preparation employed in Example 4 was about 14.

[Table 7]

Table 7: Results of Et detection in the case of acid treatment in the co-presence of a divalent metal salt

Co-present divalent metal salt	HCl concentration	Et recovery
0.5 mM ZnSO ₄	0.01 N	37.5 %
	0.05 N	66.9 %
	0.1 N	30.3 %
	0.2 N	7.9 %
	0.5 N	2.9 %
5 mM Ca(CH ₃ COO) ₂	0.01 N	11.6 %
	0.05 N	51.0 %
	0.1 N	54.6 %
	0.2 N	6.9 %
	0.5 N	3.3 %
5 mM CaCl ₂	0.01 N	14.0 %
	0.05 N	51.8 %
	0.1 N	50.2 %
	0.2 N	4.8 %
	0.5 N	2.9 %
5 mM Mg(CH ₃ COO) ₂	0.01 N	9.7 %
	0.05 N	54.9 %
	0.1 N	69.0 %
	0.2 N	No Data
	0.5 N	4.0 %
5 mM MgSO ₄	0.01 N	36.8 %
	0.05 N	59.1 %
	0.1 N	64.3 %
	0.2 N	4.9 %
	0.5 N	4.0 %
5 mM MnSO ₄	0.01 N	34.8 %
	0.05 N	48.2 %
	0.1 N	48.7 %
	0.2 N	No Data
	0.5 N	2.9 %

[0080] As shown in Table 7, through the acid treatment in the co-presence of a divalent metal, Et could be detected, similar to the case of the thermal treatment. Particularly when the hydrochloric acid concentration is 0.01 N to 0.1 N, Et could be efficiently detected.

<Example 5>

Test employing Pyrochrome (Et assay reagent for chromogenic assay)

[0081] A 0.4-EU/mL Et standard solution (0.1 mL) was added to a 50-Unit/mL AT III preparation (0.1 mL), and the mixture was vigorously agitated for one minute by means of a test tube mixer. Subsequently, water (0.1 mL) was added thereto, and the resultant mixture was vigorously agitated for one minute by means of a test tube mixer. Subsequently, a 25 mM aqueous CaCl₂ solution (0.1 mL) was added thereto, and the resultant mixture was vigorously agitated for one minute by means of a test tube mixer. An aliquot (0.2 mL) of the mixture was dispensed to another container as a 4-fold diluted solution of AT III. To the remaining portion (0.2 mL) of the mixture, water (0.2 mL) was added, and the resultant mixture was vigorously agitated for one minute by means of a test tube mixer. An aliquot (0.2 mL) of the thus-obtained diluted mixture was dispensed to another container as an 8-fold diluted solution of AT III. The operation was repeated, to thereby prepare a 16-fold diluted solution and a 32-fold diluted solution of AT III, and each diluted solution was

dispensed to another container. Each of the thus-obtained solutions was heated at 70°C for 10 minutes by means of a heat block, and then cooled with ice immediately after completion of heating. Each solution was returned to room temperature, and an aliquot (0.05 mL) thereof was dispensed to a microplate. An Et assay reagent for chromogenic assay (Pyrochrome; product of Associates of Cape Cod, Inc.) (0.05 mL) was added to the microplate, and the microplate was vigorously agitated for one minute by means of a well-reader. Thereafter, the change over time in absorbance was monitored at a measurement wavelength of 405 nm and a reference wavelength of 492 nm, while the microplate was heated at 37°C for 60 minutes, and then the Et recovery was calculated. Table 8 shows the results.

[Table 8]

Table 8: Results of Et detection through chromogenic assay (Pyrochrome)

Dilution factor	Et recovery
× 4	93.9 %
× 8	96.2 %
× 16	104.5 %
× 32	104.8 %

[0082] As shown in Table 8, even when Pyrochrome was used as an Et assay reagent for chromogenic assay instead of Endospecy ES-50 M, the Et recovery of about 100% could be attained independently of the dilution factor for the AT III preparation, i.e. Et could be detected at high accuracy. Therefore, it was elucidated that, even when Pyrochrome was used as an Et assay reagent for chromogenic assay, Et co-present with AT III could be measured at high accuracy through carrying out the pre-treatment method of the present invention.

<Example 6>

Test employing Pyrotell-T (Et assay reagent for turbidimetric assay)

[0083] A 0.4-EU/mL Et standard solution (0.1 mL) was added to a 50-Unit/mL AT III preparation (0.1 mL), and the mixture was vigorously agitated for one minute by means of a test tube mixer. Subsequently, water (0.1 mL) was added thereto, and the resultant mixture was vigorously agitated for one minute by means of a test tube mixer. Subsequently, a 25 mM aqueous CaCl₂ solution (0.1 mL) was added thereto, and the resultant mixture was vigorously agitated for one minute by means of a test tube mixer. An aliquot (0.2 mL) of the mixture was dispensed to another container as a 4-fold diluted solution of AT III. To the remaining portion (0.2 mL) of the mixture, water (0.2 mL) was added, and the resultant mixture was vigorously agitated for one minute by means of a test tube mixer. An aliquot (0.2 mL) of the thus-obtained diluted mixture was dispensed to another container as an 8-fold diluted solution of AT III. The operation was repeated, to thereby prepare a 16-fold diluted solution and a 32-fold diluted solution of AT III, and each diluted solution was dispensed to another container. Each of the thus-obtained solutions was heated at 70°C for 10 minutes by means of a heat block, and then cooled with ice immediately after completion of heating. Each solution was returned to room temperature, and an aliquot (0.1 mL) was dispensed to a microplate. An Et assay reagent for turbidimetric assay (Pyrotell (registered trademark)-T; product of Associates of Cape Cod, Inc.) (0.1 mL) was added to the microplate, and the microplate was vigorously agitated for one minute by means of a well-reader. Thereafter, the change over time in absorbance was monitored at a measurement wavelength of 405 nm and a reference wavelength of 660 nm, while the microplate was heated at 37°C for 60 minutes, and then the Et recovery was calculated. Table 9 shows the results.

[Table 9]

Table 9: Results of Et detection through turbidimetric assay (Pyrotell-T)

Dilution factor	Et recovery
× 4	99.0 %
× 8	86.2 %
× 16	80.1 %
× 32	85.5 %

[0084] As shown in Table 9, even when Pyrotell-T was used as an Et assay reagent instead of Endospecy ES-50 M, Et recovery of about 100% could be attained independently of the dilution factor for the AT III preparation, i.e. Et could

be detected at high accuracy. Therefore, it was elucidated that, even when an Et assay reagent for turbidimetric assay was used as a limulus reagent, Et co-present with AT III could be measured at high accuracy through carrying out the pre-treatment method of the present invention.

<Example 7>

Test employing Pyrotell multi-test (Et assay reagent for gel-clot assay)

[0085] A 0.24-EU/mL Et standard solution (0.1 mL) was added to a 50-Unit/mL AT III preparation (0.1 mL), and the mixture was vigorously agitated for one minute by means of a test tube mixer. Subsequently, water (0.1 mL) was added thereto, and the resultant mixture was vigorously agitated for one minute by means of a test tube mixer. Subsequently, a 25 mM aqueous CaCl_2 solution (0.1 mL) was added thereto, and the resultant mixture was vigorously agitated for one minute by means of a test tube mixer. An aliquot (0.2 mL) of the mixture was dispensed to another container as a 4-fold diluted solution of AT III. To the remaining portion (0.2 mL) of the mixture, water (0.2 mL) was added, and the resultant mixture was vigorously agitated for one minute by means of a test tube mixer. An aliquot (0.2 mL) of the thus-obtained diluted mixture was dispensed to another container as an 8-fold diluted solution of AT III. The operation was repeated, to thereby prepare a 16-fold diluted solution and a 32-fold diluted solution of AT III, and each diluted solution was dispensed to another container. Each of the thus-obtained solutions was heated at 70°C for 10 minutes by means of a heat block, and then cooled with ice immediately after completion of heating. Each solution was returned to room temperature, and an aliquot (0.1 mL) was dispensed to a round-bottom test tube. An Et assay reagent for gel-clot assay (Pyrotell (registered trademark) multi-test; product of Associates of Cape Cod, Inc.) (0.1 mL) was added to the test tube, and the resultant mixture was vigorously agitated for one minute by means of a test tube mixer, followed by heating at 37°C for one hour by means of a heat block. Thereafter, the test tube was slowly tilted, and formation of solid gel of the contents which gel did not flow out was checked. Table 10 shows the results.

[Table 10]

Table 10: Results of Et detection through gel-clot assay (Pyrotell multi-test)			
Dilution factor	Et concentration		Evaluation
× 4	0.06 EU/mL		+ (positive)
× 8	0.03 EU/mL		+ (positive)
× 16	0.015 EU/mL		- (negative)
× 32	0.0075 EU/mL		- (negative)

[0086] As shown in Table 10, gel-clot of the contents was observed in an AT III solution containing Et at a concentration of 0.03 EU/mL, which is the detection limit by Pyrotell multi-test, or higher. Therefore, it was elucidated that, even when an Et assay reagent for a gel-clot assay was used as a limulus reagent, Et co-present with AT III could be detected at high accuracy through carrying out the pre-treatment method of the present invention.

<Example 8>

Test employing parallel line assay (1)

[0087] A 1-EU/mL Et standard solution (0.11 mL) was added to a 50-Unit/mL AT III preparation (0.475 mL), and the mixture was vigorously agitated for one minute by means of a test tube mixer. Subsequently, water (0.475 mL) was added thereto, and the resultant mixture was vigorously agitated for one minute by means of a test tube mixer. Subsequently, a 500 mM aqueous MgSO_4 solution (0.05 mL) was added thereto, and the resultant mixture was vigorously agitated for one minute by means of a test tube mixer. The thus-obtained solution was heated at 70°C for 10 minutes by means of a heat block, and then cooled with ice immediately after completion of heating. The solution was returned to room temperature, and an aliquot (0.5 mL) was dispensed to another container as a twice diluted solution of AT III. To the remaining portion (0.5 mL) of the mixture, water (0.5 mL) was added, and the resultant mixture was vigorously agitated for one minute by means of a test tube mixer. An aliquot (0.5 mL) of the thus-obtained diluted mixture was dispensed to another container as a 4-fold diluted solution of AT III. The operation was repeated, to thereby prepare an 8-fold diluted solution, a 16-fold diluted solution, and a 32-fold diluted solution of AT III, and each diluted solution was dispensed to another container. Separately, water for the endotoxin test was used instead of an AT III preparation to prepare and provide a similar dilution series without the pre-treatment of the present invention as control specimens.

EP 2 940 468 A1

Each solution (0.05 mL) was dispensed to a microplate. Endospecy ES-50 M (0.05 mL) was added to the microplate, and the microplate was vigorously agitated for one minute by means of a well-reader. Thereafter, the change over time in absorbance was monitored at a measurement wavelength of 405 nm and a reference wavelength of 492 nm, while the microplate was heated at 37°C for 30 minutes. Table 11 and Fig. 1 show the results.

[Table 11]

Table 11: Results of test employing parallel line assay
(1) Measurement results of Et test water as specimen

Et concn.		Absorbance change rate (mAbs/min)			
EU/mL	log value	M1	M2	M3	Average (log value)
0.00619	-2.208	0.880	0.880	0.930	-0.048
0.01238	-1.907	1.540	1.600	1.540	0.193
0.02475	-1.606	2.970	2.970	2.970	0.473
0.04950	-1.305	6.170	5.230	5.280	0.744
0.09900	-1.004	10.150	10.120	10.110	1.005

* M1, M2, and M3: Measurement 1, 2, and 3

(2) Measurement results of AT III preparation as specimen

Dilution ratio		Absorbance change rate (mAbs/min)			
	log value	M1	M2	M3	Average (log value)
0.02675	-1.573	1.020	1.020	1.020	0.009
0.05350	-1.272	1.750	1.760	1.930	0.258
0.10700	-0.971	3.360	3.280	3.230	0.517
0.21400	-0.670	6.520	6.650	6.650	0.820
0.42800	-0.369	12.090	13.320	12.670	1.103

* M1, M2, and M3: Measurement 1, 2, and 3

[0088] As shown in Fig. 1, the measured absorbance change rates of AT III-containing specimens were log-log plotted with respect to the dilution factors, and the measured absorbance change rates of control specimens (twice dilution series of endotoxin standard solution with water for endotoxin test) were log-log plotted with respect to endotoxin concentrations. The thus-plotted two lines were tested in terms of parallelism through a parallel line assay. The assay was performed through statistical analysis by use of software specialized to the parallel line assay (PL603; product of Seikagaku Corporation). As a result, regression linearity was established in each line, and parallelism was established between the two lines. Thus, the test results were satisfactory. The Et recovery, calculated by dividing the Et concentration (0.275 EU/mL) calculated through the parallel line assay by the Et concentration of the control (0.232 EU/mL), was 118.5%.

<Example 9>

Test employing parallel line assay (2)

[0089] A 1-EU/mL Et standard solution (0.1 mL) was added to a 50-Unit/mL AT III preparation (0.8 mL), and the mixture was vigorously agitated for one minute by means of a test tube mixer. Subsequently, water (0.02 mL) was added thereto, and the resultant mixture was vigorously agitated for one minute by means of a test tube mixer. Subsequently, a 500 mM aqueous MgSO₄ solution (0.08 mL) was added thereto, and the resultant mixture was vigorously agitated for one minute by means of a test tube mixer. The thus-obtained solution was heated at 70°C for 10 minutes by means of a heat block, and then cooled with ice immediately after completion of heating. The solution was returned to room temperature, and an aliquot (0.5 mL) was dispensed to another container as a 1.25-fold diluted solution of AT III. To the remaining portion (0.5 mL) of the mixture, 40 mM aqueous MgSO₄ (0.5 mL) was added, and the resultant mixture was vigorously agitated for one minute by means of a test tube mixer. An aliquot (0.5 mL) of the thus-obtained diluted mixture was dispensed to another container as a 2.5-fold diluted solution of AT III. The operation was repeated, to thereby prepare a 5-fold diluted solution, a 10-fold diluted solution, and a 20-fold diluted solution of AT III, and each diluted solution was dispensed to another container. Separately, water for the endotoxin test was used instead of an AT III preparation to

prepare and provide a similar dilution series without the pre-treatment of the present invention as control specimens. Each solution (0.05 mL) was dispensed to a microplate. Endospecy ES-50 M (0.05 mL) was added to the microplate, and the microplate was vigorously agitated for one minute by means of a well-reader. Thereafter, the change over time in absorbance was monitored at a measurement wavelength of 405 nm and a reference wavelength of 492 nm, while the microplate was heated at 37°C for 30 minutes. Table 12 and Fig. 2 show the results.

[Table 12]

(1) Measurement results of Et test water as specimen				
Et concn.		Absorbance change rate (mAbs/min)		
EU/mL	log value	M1	M2	Average (log value)
0.00625	-2.204	0.890	0.950	-0.036
0.01250	-1.903	1.660	1.430	0.188
0.02500	-1.602	3.270	3.190	0.509
0.05000	-1.301	5.950	6.050	0.778
0.10000	-1.000	11.460	11.710	1.064
* M1 and M2: Measurement 1 and 2				
(2) Measurement results of AT III preparation as specimen				
Dilution ratio		Absorbance change rate (mAbs/min)		
	log value	M1	M2	Average (log value)
0.05000	-1.301	0.930	0.910	-0.036
0.10000	-1.000	1.690	1.690	0.228
0.20000	-0.699	3.040	2.960	0.477
0.40000	-0.398	5.560	5.650	0.749
0.80000	-0.097	10.600	10.970	1.033
* M1 and M2: Measurement 1 and 2				

[0090] As shown in Fig. 2, the measured absorbance change rates of AT III-containing specimens were log-log plotted with respect to the dilution factors, and the measured absorbance change rates of control specimens (twice dilution series of endotoxin standard solution with water for endotoxin test) were log-log plotted with respect to endotoxin concentrations. The thus-plotted two lines were tested in terms of parallelism through a parallel line assay. The assay was performed through statistical analysis by use of software specialized to the parallel line assay (PL603; product of Seikagaku Corporation). As a result, regression linearity was established in each line, and parallelism was established between the two lines. Thus, the test results were satisfactory. The Et recovery, calculated by dividing the Et concentration (0.122 EU/mL) calculated through the parallel line assay by the Et concentration of the control (0.125 EU/mL), was 97.6%.

<Example 10>

Test employing parallel line assay (3)

[0091] A 20-EU/mL Et standard solution (2.5 μ L) was added to a 50-Unit/mL AT III preparation (472.5 μ L), and the mixture was vigorously agitated for one minute by means of a test tube mixer. Subsequently, water (6.25 μ L) was added thereto, and the resultant mixture was vigorously agitated for one minute by means of a test tube mixer. Subsequently, a 2M aqueous $MgSO_4$ solution (18.75 μ L) was added thereto, and the resultant mixture was vigorously agitated for one minute by means of a test tube mixer. The thus-obtained solution was heated at 70°C for 20 minutes by means of a heat block, and then cooled with ice immediately after completion of heating. The solution was returned to room temperature, and an aliquot (250 μ L) was dispensed to another container as a 1.06-fold diluted solution of AT III. To the remaining portion (250 μ L) of the mixture, water (250 μ L) was added, and the resultant mixture was vigorously agitated for one minute by means of a test tube mixer. An aliquot (250 μ L) of the thus-obtained diluted mixture was dispensed to another container as a 2.12-fold diluted solution of AT III. The operation was repeated, to thereby prepare a 4.23-fold diluted solution, an 8.47-fold diluted solution, and a 16.93-fold diluted solution of AT III, and each diluted solution was dispensed to another container. Separately, water for the endotoxin test was used instead of an AT III preparation to prepare and

provide a similar dilution series without the pre-treatment of the present invention as control specimens. Each solution (50 μ L) was dispensed to a microplate. Endospecy ES-50 M (50 μ L) was added to the microplate, and the microplate was vigorously agitated for one minute by means of a well-reader. Thereafter, the change over time in absorbance was monitored at a measurement wavelength of 405 nm and a reference wavelength of 492 nm, while the microplate was heated at 37°C for 30 minutes. Table 13 and Fig. 3 show the results.

[Table 13]

(1) Measurement results of Et test water as specimen				
Et concn.		Absorbance change rate (mAbs/min)		
EU/mL	log value	M1	M2	Average (log value)
0.00625	-2.204	0.860	0.880	-0.061
0.01250	-1.903	1.460	1.420	0.158
0.02500	-1.602	2.600	2.520	0.408
0.05000	-1.301	5.050	4.970	0.700
0.10000	-1.000	9.400	9.580	0.977
* M1 and M2: Measurement 1 and 2				
(2) Measurement results of AT III preparation as specimen				
Dilution ratio		Absorbance change rate (mAbs/min)		
	log value	M1	M2	Average (log value)
0.05906	-1.229	0.920	0.930	-0.034
0.11813	-0.928	1.510	1.620	0.194
0.23625	-0.627	2.870	3.210	0.482
0.47250	-0.326	4.580	5.540	0.702
0.94500	-0.025	7.960	8.640	0.919
* M1 and M2: Measurement 1 and 2				

[0092] As shown in Fig. 3, the measured absorbance change rates of AT III-containing specimens were log-log plotted with respect to the dilution factors, and the measured absorbance change rates of control specimens (twice dilution series of endotoxin standard solution with water for endotoxin test) were log-log plotted with respect to endotoxin concentrations. The thus-plotted two lines were tested in terms of parallelism through a parallel line assay. The assay was performed through statistical analysis by use of software specialized to the parallel line assay (PL603; product of Seikagaku Corporation). As a result, regression linearity was established in each line, and parallelism was established between the two lines. Thus, the test results were satisfactory. The Et recovery, calculated by dividing the Et concentration (0.111 EU/mL) calculated through the parallel line assay by the Et concentration of the control (0.106 EU/mL), was 104.7%.

[0093] Thus, as shown in Examples 8 to 10, through performing the pre-treatment of the present invention, Et co-present with AT III can be measured at high accuracy, even in the test according to the parallel line assay described in the biological preparation standards.

Industrial Applicability

[0094] According to the present invention, by performing a pre-treatment of AT III to be subjected to limulus test, reaction interference observed when AT III is subjected to limulus test can be reduced, and thus, limulus test of AT III can be carried out at high accuracy. Also, according to the present invention, limulus test of AT III is expected to be carried out in a simple and speedy manner at low cost.

SEQUENCE LISTING

<110> SEIKAGAKU CORPORATION

<120> Pretreatment agent and pretreatment method for antithrombin III to be subjected to limulus test

<130> F201302-3372

<150> JP2012-287475

<151> 2012-12-28

<160> 1

<170> PatentIn version 3.5

<210> 1

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> peptide

<400> 1

Ile Glu Gly Arg

1

Claims

1. A pre-treatment agent for antithrombin III to be subjected to limulus test, which agent contains a divalent metal salt, and which agent is used for subjecting antithrombin III to a protein inactivation treatment in the co-presence of the pre-treatment agent, before antithrombin III is subjected to limulus test.
2. The pre-treatment agent according to claim 1, wherein the divalent metal salt is one or more metal salts selected from the group consisting of a divalent metal chloride, a divalent metal acetate, and a divalent metal sulfate.
3. The pre-treatment agent according to claim 1 or 2, wherein the divalent metal constituting the divalent metal salt is one or more metals selected from the group consisting of magnesium, calcium, manganese, and zinc.
4. The pre-treatment agent according to any one of claims 1 to 3, wherein the concentration of the divalent metal ion derived from the divalent metal salt is 0.5 mM or higher when antithrombin III is co-present with the pre-treatment agent.
5. The pre-treatment agent according to any one of claims 1 to 4, wherein a target substance of the limulus test is endotoxin.
6. A limulus reagent kit for antithrombin III, the kit comprising the pre-treatment agent according to any one of claims 1 to 5.
7. A pre-treatment method for antithrombin III to be subjected to limulus test, the method comprising subjecting antithrombin III to a protein inactivation treatment in the co-presence of the pre-treatment agent according to any one of claims 1 to 5.
8. The pre-treatment method according to claim 7, wherein the protein inactivation treatment is a thermal treatment or an acid treatment.

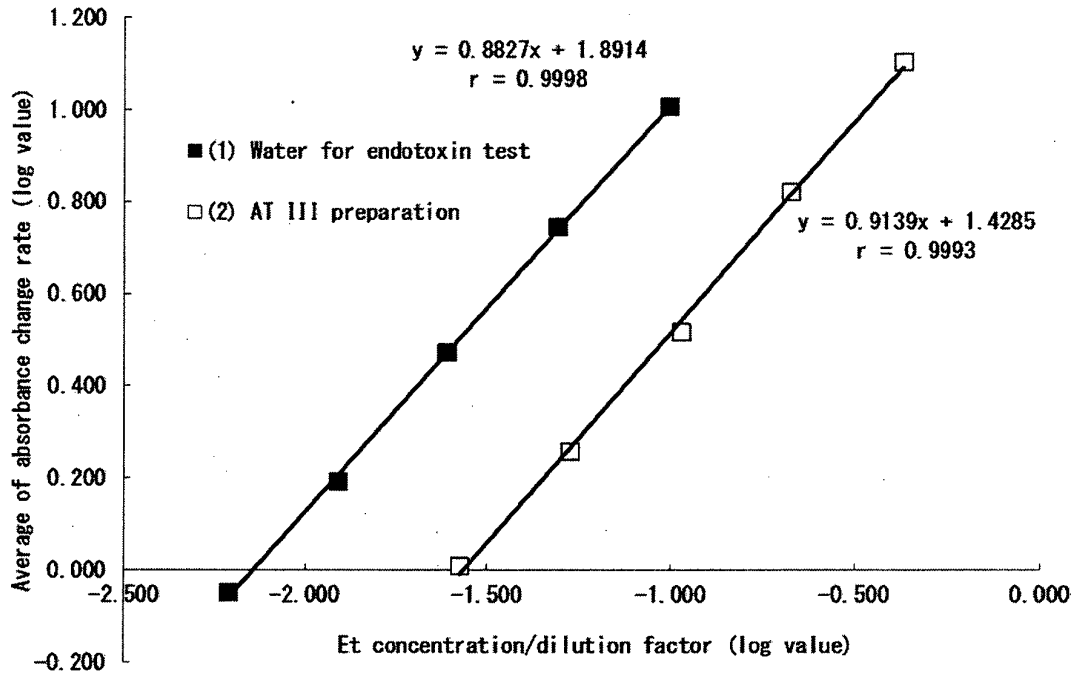
9. The pre-treatment method according to claim 8, wherein the thermal treatment is performed at a temperature higher than 50°C.

10. The pre-treatment method according to claim 8, wherein the acid employed in the acid treatment is hydrochloric acid.

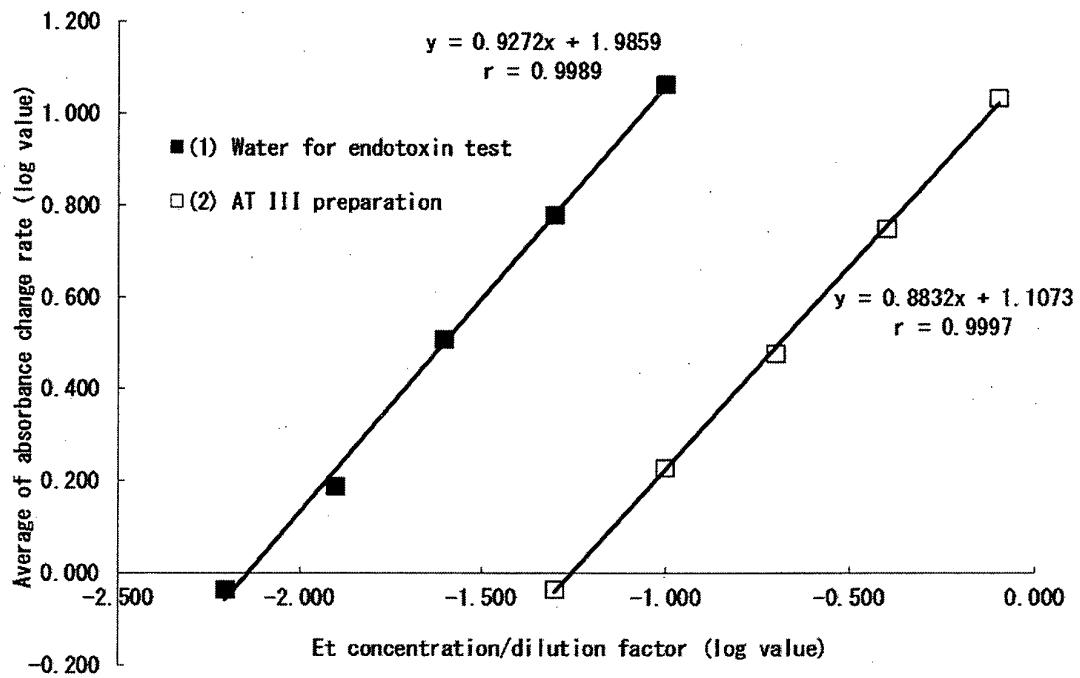
11. A method for measuring a target substance of limulus test contained in antithrombin III, the method comprising preliminarily treating antithrombin III through the pre-treatment method according to any one of claims 7 to 10, and subjecting the preliminarily treated antithrombin III to limulus test.

12. A method for producing antithrombin III, the method comprising measuring the target substance of limulus test in antithrombin III through the method according to claim 11.

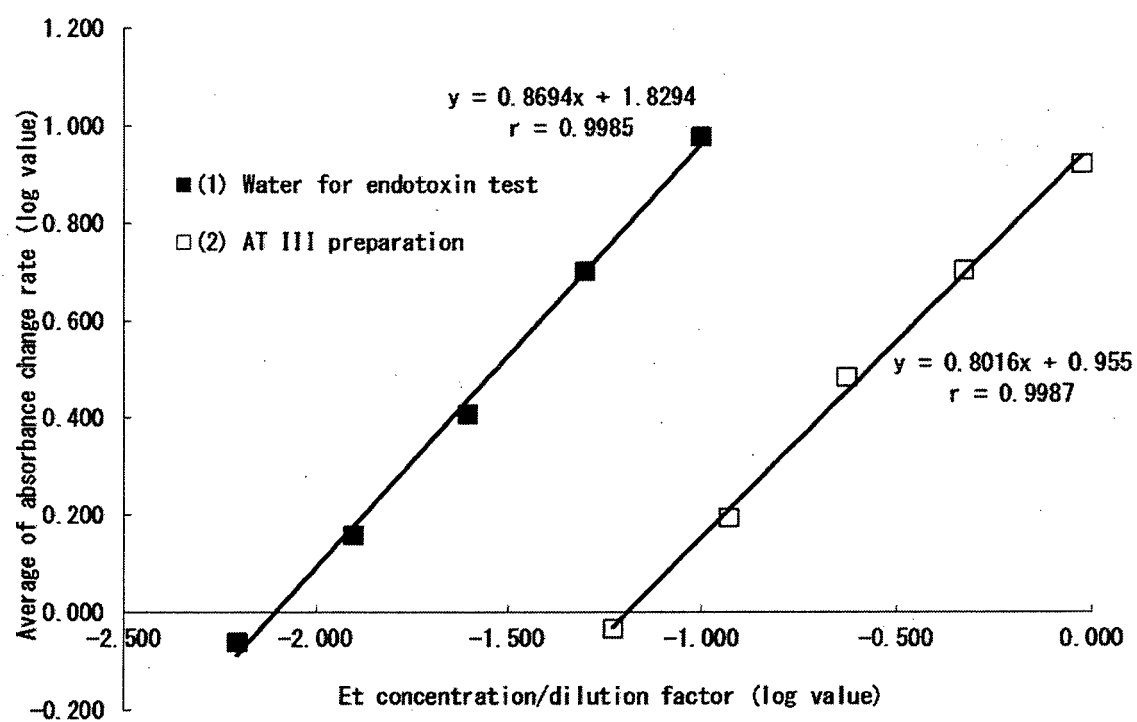
[Fig. 1]



[Fig. 2]



[Fig. 3]



INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2013/085234

A. CLASSIFICATION OF SUBJECT MATTER

G01N33/48(2006.01)i, G01N33/579(2006.01)i, C07K5/103(2006.01)n

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N33/48, G01N33/579, C07K5/103

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2014

Kokai Jitsuyo Shinan Koho 1971-2014 Toroku Jitsuyo Shinan Koho 1994-2014

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

JSTPlus/JMEDPlus/JST7580(JDreamIII)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A/Y	JP 09-178753 A (Masashi FUNAYAMA), 11 July 1997 (11.07.1997), entire text (Family: none)	1-9, 11, 12/10

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

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Date of the actual completion of the international search

18 March, 2014 (18.03.14)

Date of mailing of the international search report

01 April, 2014 (01.04.14)

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2013/085234

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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A	JP 2004-109147 A (Seikagaku Corp.), 08 April 2004 (08.04.2004), entire text & JP 6-70796 A & US 5389547 A & EP 569033 A2 & DE 69325848 C & DE 69325848 T & CN 1087724 A	1-12
A	JP 2010-243342 A (Peptide Door Co., Ltd.), 28 October 2010 (28.10.2010), entire text (Family: none)	1-12
A	JP 04-102064 A (Seikagaku Corp.), 03 April 1992 (03.04.1992), entire text & US 5286625 A & US 5605806 A & EP 500947 A1 & WO 1992/003736 A1 & DE 69129365 C & DE 69129365 D	1-12

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